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Effects of Nicotine and Tocotrienol-Rich Fraction Supplementation on Cytoskeletal Structures of Murine Pre-Implantation Embryos

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Background: Cytoskeletal structures, in particular actin and tubulin, provide a fundamental framework in all cells, including embryos. The objective of this study was to evaluate the effects of nicotine, which is a source of oxidative stress, and subsequent supplementation with Tocotrienol-rich fraction (TRF) on actin and tubulin of 2- and 8-cell murine embryos.

Material/Methods: Thirty female Balb/C mice were divided into 4 groups: Group 1 received: subcutaneous (sc) injection of 0.9% NaCl; Group 2 received sc injection of 3.0 nicotine mg/kg bw/day; Group 3 received 3.0 sc injection of nicotine mg/kg bw/day +60 mg/kg bw/day TRF; and Group 4 received 60 sc injection of TRF mg/kg bw/day for 7 consecutive days. The animals were superovulated with 5 IU PMSG followed by 5 IU hCG 48 h later. Animals were cohabited with fertile males overnight and euthanized through cervical dislocation at 24 h post coitum. Embryos at the 2- and 8-cell stages were harvested, fixed, and stained to visualize actin and tubulin distributions by using CLSM.

Results: Results showed that at 2-cell stage, actin intensities were significantly reduced in the nicotine group compared to that of the control group ($p<0.001$). In Group 3, the intensity of actin significantly increased compared to that of the nicotine group ($p<0.001$). At 8-cell stage, actin intensity of the nicotine group was significantly lower than that of the control group ($p<0.001$). The intensities of actin in Group 3 were increased compared to that of nicotine treatment alone ($p<0.001$). The same trend was seen in tubulin at 2- and 8-cell stages. Interestingly, both actin and tubulin structures in the TRF-treated groups were enhanced compared to the control.

Conclusions: This study suggests that TRF prevents the deleterious effects of nicotine on the cytoskeletal structures of 2- and 8-cell stages of pre-implantation mice embryos *in vitro*.

MeSH Keywords: Actin Cytoskeleton • Nicotine • Tocotrienols • Tubulin

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Background

Smoking during pregnancy has been associated with a higher rate of infertility, pregnancy difficulties, and damage to the developing embryos. Studies have shown that maternal smoking during pregnancy decreases fetal growth and increases low birth weight and preterm births [1–3]. Spontaneous abortion has also been reported to be significantly increased in smokers *vs.* non-smokers [4].

As a main component of tobacco smoke, nicotine contributes about 90–95% of total tobacco alkaloids and it is the most important pharmacologically active compound in tobacco smoke [5]. Nicotine penetrates the placenta and enters the fetal circulation, and hence may disturb fetal development. It also causes changes in DNA methylation of genes associated with growth restriction (e.g., CYP1A1 promoter) [6–8]. Thus, it is responsible for a wide variety of negative reproductive outcomes [9,10]. In terms of oxidative stress (OS), nicotine through maternal cigarette smoking increases OS in both mother and fetus [11]. Reactive oxygen species (ROS), which are initiators of OS, can induce many cellular damages such as oxidation and fragmentation of proteins, peroxidation of membrane lipids, fragmentation of DNA, mitochondrial damage, and disruption of ion homeostasis [12,13]. Cigarette smoke contains many toxic substances and pro-oxidants that can produce ROS, for example, the SO^{-} anion, H_2O_2, and the OH^{-} radical, which have been reported to cause damage to the membrane structure at chromosomal [14] and ultrastructural levels [15]. Until now, reports on the effects of nicotine on the cytoskeletal structure of pre-implantation embryo in mice are lacking. Further studies on the subsequent effect of TRF on the embryos from nicotine-induced mice are also not available.

The cytoskeleton comprises 2 major protein filaments: microfilaments (MFs) and microtubules (MTs). The MFs are composed of a protein, actin, and the MTs consist of subunits of protein, tubulin. They are accessory proteins that aid in cytoskeletal assembly, disassembly, stability, and cellular transport. The cytoskeleton is important in cell functions and embryonic development [16]; it gives support to the cell membrane, helps evenly split up chromosomes during cell division, and is also involved in organelle trafficking, which is the movement of cell components [17,18]. The cytoskeleton has the ability to alter the membrane [19] and provides a basis for movement and cell division [20,21].

Actin filaments are extremely dynamic and play a major role in providing an important cytoskeletal framework in all cells. Actin is also involved in cytokinesis and cellular movements, whereas tubulin structures are involved in the transport of cellular materials and dividing chromosomes during cell division. Therefore, toxicants that alter and disrupt the cytoskeleton (e.g., nicotine) initiate early events in cell injury and cause the nonviability of the cell, thus cell development and survivability.

Previous studies on the deleterious effects of nicotine on preimplantation embryos have implicated the role of ROS as the major factor [22]. Therefore, it is logical to study the effect of supplementation of an antioxidant on nicotine-induced embryos. One of the antioxidants is vitamin E, which exists in 2 forms: tocopherol and tocotrienol. While vitamin E is commonly referred to as α-tocopherol, the role of tocotrienols is often underrated. Tocotrienol is an important supplement in nutrition with benefits in the prevention of cancer [23] and reduction of cardiovascular disease risk by lowering total cholesterol and low-density lipoprotein (LDL) [24]. It also has neurodegenerative and neuroprotective abilities [25], as well as benefits for bone formation and repair bone damage caused by long-term smoking [26,27] Our previous studies reported that tocotrienol was able to reverse the OS effects on pre-implantation mouse embryos induced by corticosterone [28,29] and fetal development and survivability [30]. A study by Mokhtar et al. [31] showed that oral supplementation with tocotrienol increased the percentage of pregnancy outcome. Rajikin et al. [15] found effects of nicotine and supplementation on preovulation oocytes, although such studies in pre-implantation embryos are lacking. The present study investigated the effects of nicotine and the subsequent supplementation of TRF on the cytoskeletal structure (i.e., actin and tubulin).

Although a number of studies have been carried out to determine the role of tocotrienol in pre-implantation embryo development, there are few reports on the role of this antioxidant on cytoskeletal structures of the embryos exposed to OS.

Immunofluorescence staining and microscopy techniques were used to examine cellular structures for actin and tubulin. A confocal laser scanning microscope (CLSM) was used to measure the intensities of cytoskeletal structures of pre-implantation embryo in mice induced with nicotine. Visual images of the cytoskeletal structure disruption that occurs with nicotine convey the dangerous effects of cigarette smoke during pregnancy, and the beneficial effect of TRF on OS suggest a potential prevention mechanism.

Material and Methods

Experimental animals and treatment

Twenty-four female mice from Balb/C strain aged 5–6 weeks and weighing 15–16 g were randomly divided into 4 groups. The palm oil TRF (Gold Tri.E 70), containing 75% tocotrienol and 25% tocopherol was purchased from Sime-Darby. All treatments were conducted between 8 am and 10 am for 7

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consecutive days. The first group received 0.9% NaCl, subcutaneously (sc). The second group received 3.0 mg/kg bw/day nicotine (sc). The third group received 3 mg/kg bw/day nicotine (sc) followed by 60 mg/kg bw/day TRF oral gavage. The forth group received 60 mg/kg bw/day TRF oral gavage. All animals were kept in standard laboratory conditions at 27°C with 12-h light-dark periods and were given food pellets and water *ad libitum*. Ethics approval from the university Animal Care and Use Committee was obtained (ACUC 101/2015). All procedures followed the institutional animal ethics guidelines.

Origin, culture and harvest of embryos

Female mice were superovulated (intraperitoneal; ip) using pregnant mare's serum gonadotropin (PMSG) (5 IU/kg bw) followed by human chorionic gonadotropin (hCG) (5 IU/kg bw) hormones (Intervet, Holland) 48 h post-PMSG before being mated with fertile male mice at a ratio of 1: 1. Female mice with the presence of vaginal plugs were considered to be pregnant and were selected as embryo donors. The mice were then euthanized by cervical dislocation 48 h after copulation. Fallopian tubes were excised and embryos were flushed under a dissecting microscope (Leica Zoom 2000, Japan). The 2-cell embryos were rinsed with M2 medium and cultured *in vitro* (37°C CO_2 incubator [5% CO_2; 95% air]) in 24-well plates (Orange Scientific, Belgium) filled with 100 µl of M16 medium (Sigma, USA) overlaid with mineral oil (Sigma, USA), until the embryos developed into the 8-cell stage. Development of embryos was observed daily under an inverted microscope (Olympus 1X81 SF-3, Japan).

Cytoskeletal staining

A total of 500 embryos at 2- and 8-cell stages were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h at room temperature. The fixed cells were then incubated with 4',6-diamino-2-phenylindole dihydrochloride (DAPI) (300 nM in PBS) for 40 min to stain the nucleus before being permeabilized with 0.1% Triton X-100 in PBS for 10 min. Alexa Fluor® 635 phalloidin (6.6 µM in PBS) was added to the permeabilized cells and incubated for 1 h to stain the actin structure, and 1 µl of anti-α-tubulin conjugated antibody was diluted in 49 µl of Abdil (with ratio 1: 50) to stain the tubulin structures. The embryos were washed with PBS twice for 10 min each. Embryos were mounted on slides with Prolong® Gold Antifade reagent to minimize photo bleaching and stored at 4°C in the dark, viewed under CLSM (Leica TCS SP5). Images taken from CLSM were analyzed using LAS AF Lite software. The fluorescence intensities of the actin (excitation: 633 nm, emission: 647 nm) structures were measured according to their wavelengths.

Statistical analysis

Each experimental arm comprised 50 embryos for each stages of development. Assessment of cytoskeletal quality was based on the intensity of fluorescent probes after immunofluorescence staining and the morphology of the embryos. The percentage of embryo development at 2- and 8-cell stages indicates the survivability of the embryos. Intensities of fluorescent probes in each stage of embryo development were compared between experimental groups using the independent-samples *t* test. All data are expressed as mean ± SEM. In all cases, statistical analyses were performed using the Statistical Package for Social Sciences, version 20 (SPSS Inc, USA), and the differences were considered significant when $p<0.05$.

Results

The differences in the distribution of actin in pre-implantation embryos in all 4 groups were captured by using CLSM. Red staining signifies the actin structures of embryos stained with Alexa Fluor 635 phalloidin.

Results on the distribution of actin are summarized in Figures 1 and 2. At the 2-cell stage, actin intensities were significantly reduced in the nicotine group (12.3±0.1) (Figure 1B) compared to that of the control group (41.9±0.8) (Figure 1A), ($p<0.001$). Intervention with TRF (Nicotine + TRF group) (32.6±2.0) (Figure 1C) significantly reversed the intensity of actin compared to that of the nicotine group (12.3±0.1) (Figure 1B), ($p<0.001$). When the animals were given TRF alone (TRF group), actin intensity was higher (57.7±1.1) (Figure 1D) compared to that of nicotine + TRF group (32.6±2.0) (Figure 1C), ($p<0.001$).

The same trend was seen as the embryo developed further to the 8-cell stage. Actin intensity of the nicotine group (9.2±0.6) (Figure 1F) was significantly lower than that of the control group (50.1±2.5) (Figure 1E), ($p<0.001$). The intensities of actin in nicotine + TRF group was increased and higher (40.3±0.8) (Figure 1G) compared to that of the nicotine-treated alone group (9.2±0.6) (Figure 1F), ($p<0.001$). Actin intensities were significantly increased in TRF treatment (60.2±1.9) (Figure 1H) compared to that of the nicotine + TRF treatments (40.3±0.8) (Figure 1G), ($p<0.001$).

The tubulin structure stained with anti-α-tubulin is shown in Figures 3 and 4. At the 2-cell stage, tubulin intensities were significantly reduced in the nicotine-treated group (13.7±1.1) (Figure 3B) compared to that of the control group (55.2±1.1), ($p<0.001$) (Figure 3A). The intensities of tubulin were significantly reversed in the nicotine + TRF group compared to that of the nicotine group [(nicotine + TRF group *vs.* nicotine group; 44.3±1.2 *vs.* (13.7±1.1) ($p<0.001$) (Figure 3C, 3B)]. Results also

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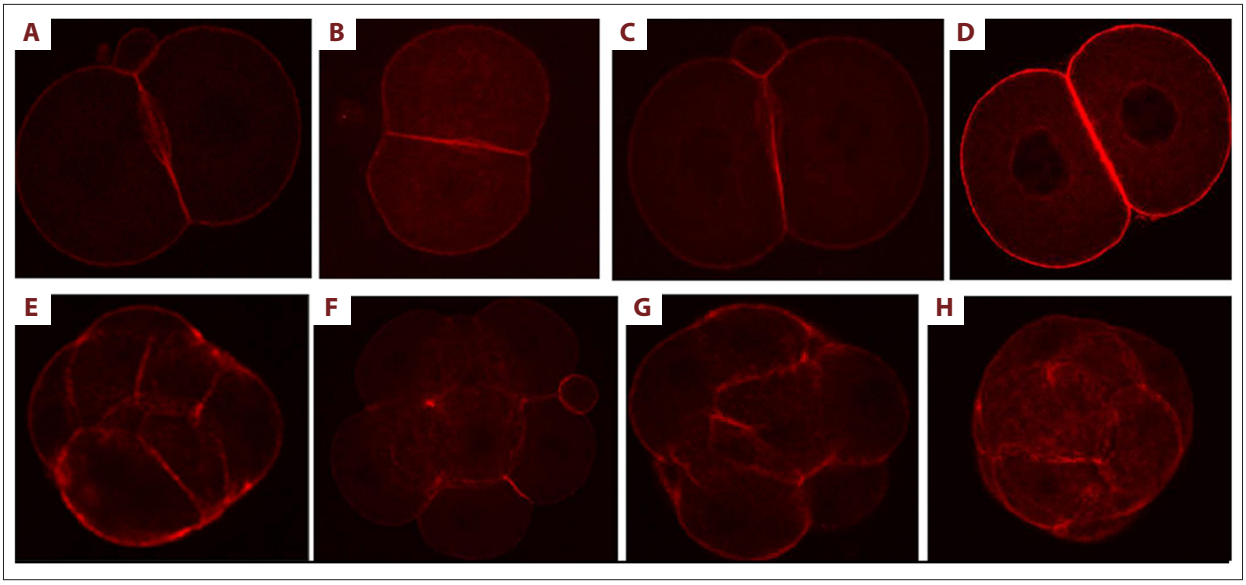

Figure 1. Immunofluorescent intensities of actin at 2-cell (**A–D**) and 8-cell (**E–H**) stages of embryos. Control group (**A, E**), nicotine group (**B, F**), nicotine + TRF group (**C, G**) and TRF group (**D, H**) embryos stained with Alexa Fluor 635 phallodin for actin (red) were observed under a CLSM.

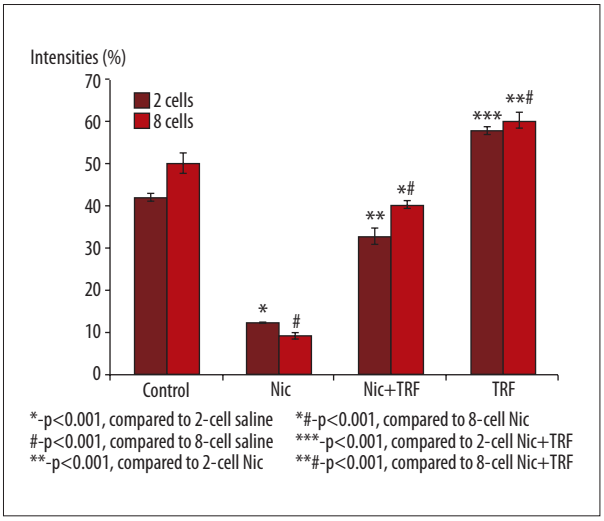

Figure 2. Comparisons of actin intensity (%) between all treatment groups. Values with different superscripts are significantly different. Nic – Nicotine, TRF – tocotrienol-rich.

showed that tubulin intensities were significantly higher in the TRF treatment (86.±1.2) (Figure 3D) compared to that of the nicotine + TRF treatment (44.3±1.2), (p<0.001) (Figure 3C).

At the 8-cells stage, the cortex area became strongly stained with anti-α-tubulin and perinuclear clustering was observed in all groups, but not in the nicotine group. In the nicotine group, the tubulin intensity was significantly lower (4.2±0.7) (Figure 3F) compared to that of the control group 70.7±3.7) (Figure 3E), (p<0.001). Intervention with TRF after nicotine treatment (nicotine + TRF group) (58.4±2.5) (Figure 3G) significantly increased the intensity of tubulin compared to that of nicotine group (4.2±0.7), (p<0.001) (Figure 3F). In the group given TRF alone, the distribution of tubulin was higher (97.2±0.6) (Figure 3H) compared to that of the nicotine + TRF group (58.4±2.5) (p<0.001) (Figure 3G).

Discussion

The effects of nicotine and TRF intervention on embryonic cytoskeletal structures of actin and tubulin were studied at 2- and 8-cell stages of pre-implantation embryos. Confocal laser scanning microscope (CLSM) images showed that nicotine caused significant decrease in the fluorescent intensities of actin and tubulin at the 2- and 8-cell stages.

Actin and tubulin are very sensitive, unique and fragile structures and play major roles in normal function and development of cells [32]. Nicotine has been shown to induce oxidative stress, and exposure to nicotine may affect the cell proliferation and differentiation during embryonic development [33]. Results of the present study suggest that the reduction in the fluorescent intensity may reflect cytoskeletal damage, which may affect the embryo development later. Results from this study also proved that nicotine caused damage to the intracellular network of actin and tubulin that are essential for cytokinesis and karyokinesis. Damage to actin and tubulin may disrupt the cell cycle and alter cell viability. The exact mechanism by which nicotine causes damage to the cytoskeletal structure is unknown. However, nicotine has been proven

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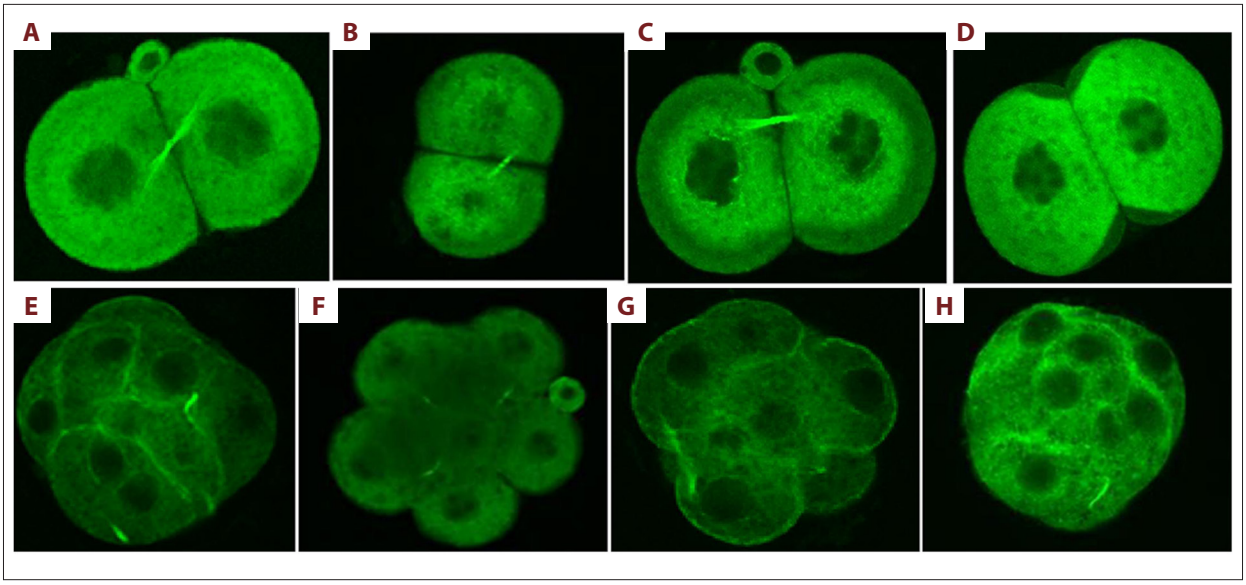

Figure 3. Immunofluorescent intensities of tubulin at 2-cell (**A–D**) and 8-cell (**E–H**) stages of embryos. Control group (**A, E**), nicotine group (**B, F**), nicotine + TRF group (**C, G**) and TRF group (**D, H**) embryos stained with anti-α-tubulin for tubulin (green) were observed under a CLSM.

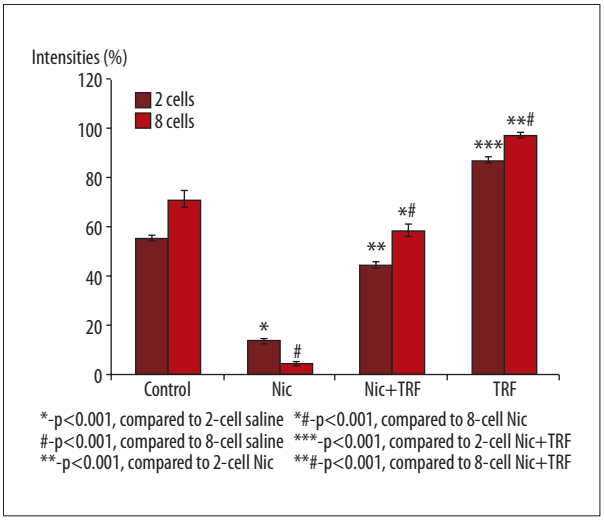

Figure 4. Comparisons of tubulin intensity (%) between all treatment groups. Values with different superscripts are significantly different. Nic – Nicotine, TRF – tocotrienol-rich.

to elevate plasma MDA, an indicator of oxidative stress [33]. Although the present study did not measure the OS status of animals, it is possible that OS induced by nicotine causes alteration in the transmembrane protein attached to the cytoskeletal structures.

Results from this study also showed the ability of TRF to effectively reverse the deleterious effects of nicotine in 2- and 8-cell embryos, as was indicated by the higher intensities of actin and tubulin. Approximately 75% of tocotrienol in TRF is responsible for the observed benefits, and the presence of 3 trans double bonds in tocotrienol in contrast with the saturated side chain of tocopherol makes tocotrienol more readily transferred and incorporated into the cell membranes, which may aid in the repair of actin and tubulin structures. The biodistribution study of tocotrienol in null rodents showed that tocotrienol could be delivered to all organs [34]. Tocotrienol are deposited in the adipose tissues, skin, and heart, suggesting that tocotrienol is absorbed and distributed *in vivo* [35,36]. The additional 3 double bonds give tocotrienol greater fluidity and more mobility in the cellular membrane, which gives it specific biological and therapeutic properties compared to tocopherol [37].

The have been no similar studies on the effect of nicotine and subsequent supplementation of TRF. However, studies on actin and tubulin in cryopreserved embryos by the slow freezing method have shown that the procedure caused damage to actin and tubulin [38]. Whether the nature of the damage caused by cryopreservation is similar to that of nicotine warrants further investigation. Our results showed that supplementation with 60 mg/kg bw/day TRF is sufficient in scavenging the free radical following nicotine exposure. The present results were similar to those of a previous study done by our group, which compared 3 different doses of gamma-tocotrienol (γ-TCT) – 30 mg/kg bw/day, 60 mg/kg bw/day, and 90 mg/kg bw/day – showing that supplementation with 60 mg/kg bw/day γ-TCT was the optimal dose that maintains *in vitro* embryonic development in nicotine-induced oxidative stress [30]. It is interesting that the distribution of actin and tubulin was highest when mice were treated with TRF alone.

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In the present study, we investigated 2 stages of pre-implantation embryonic development: 2- and 8-cell stages. These 2 stages reflect the variations of early stages of embryo development. The 2-cell embryos depend on their maternal factors to maintain their integrity in cellular activities. The first role of maternal factors is the processing of the male genome, which is important in embryogenesis; the second role is the elimination of maternal detritus (RNA and protein); and the third role is to activate the embryonic genome, which is important for the development of the embryo beyond 2-cell stage [39]. On the other hand, activation of the mouse embryonic genome occurs at 8-cell stage. The intensities of actin and tubulin distribution in all groups except that in the nicotine group were higher in 8-cells compared to those in 2-cells. This holds true for both actin and tubulin, and could be due to the increase in cell numbers.

Despite the finding of actin and tubulin damage following nicotine and the reversal following TRF supplementation, it is yet to be ascertained whether the numerous toxic constituents in nicotine adversely affect the cell proliferation and differentiation during embryonic development. However, a study by Syairah et al. [40] showed a significant aberration in the embryonic DNA samples following treatment with nicotine. The present findings raise questions about whether some signalling pathways or molecular mechanisms are involved behind the reported results. Therefore, future research should include comprehensive assessment of related signalling pathways and mechanisms involved.

Conclusions

Nicotine was the source of ROS that caused deleterious effects on actin and tubulin. Although measurements of OS biomarkers are not reported here, previous studies have shown that ROS originates from exogeneous nicotine and increases malondialdehyde (MDA) level, and subsequently caused damage to the cells. This study also showed that TRF may be used to improve development of nicotine-exposed embryos, and, surprisingly, suggests that TRF alone may enhance development of normal embryos. Further specific molecular data are required before TRF can be suggested as a therapeutic agent to improve embryonic cellular organization.

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